# Polyoxazoline Thermoresponsive Micelles as Radionuclide Delivery Systems[a]

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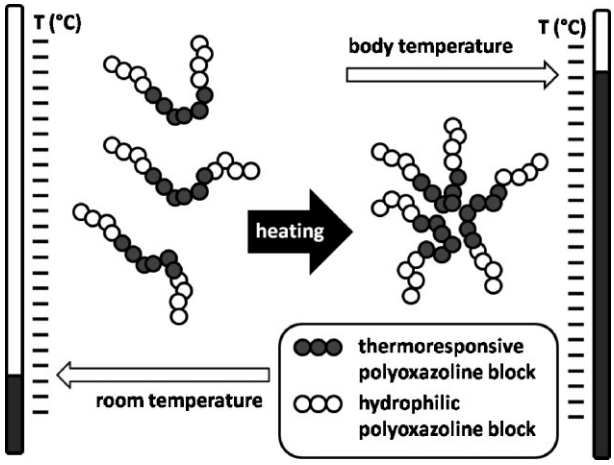
Thermoresponsive polymer micelles are promising drug and radionuclide carriers with a strong passive targeting effect into solid tumors. We have synthesized ABA triblock copolymers poly[2-methyl-2-oxazoline-*block*-(2-isopropyl-2-oxazoline-*co*-2-butyl-2-oxazoline)-*block*-2-methyl-2-oxazoline]. These polymers are molecularly dissolved in aqueous millieu below the cloud point temperature (CPT) of the thermoresponsive central block and above CPT form polymer micelles at CMC 5–10 $\times 10^{-5}$ g $\cdot$ mL$^{-1}$ with diameter ≈200 nm. The phenolic moiety introduced into the copolymer allowed radionuclide labeling with iodine-125 ongoing in good yield with sufficient in vitro stability under model conditions.




## Introduction

Poly(2-alkyl-2-oxazolines) are attracting increasing attention in biomedicinal research due to their peptide-related structure and wide range and adjustable physicochemical and biological properties depending on the alkyl substituent.[1–3] Their properties range from high hydrophilicity enabling synthesis of hydrophilic water soluble biocompatible polymers with excellent antibiofouling properties (alkyl = methyl or ethyl)[4] through thermal sensitivity of thermoresponsive polymers (alkyl = isopropyl)[2,5] to hydrophobicity typical for hydrophobic aromatic or aliphatic polymers (substituent = phenyl, butyl, nonyl etc.).[1,6] Moreover, polyoxazolines may be synthesized by controlled living cationic ring-opening polymerization with polydispersities typically below 1.3 and this method enables synthesis of block copolymers by subsequent addition of different monomers.[7,8] The choice of initiator and terminating agent also enables synthesis of polymers with defined chain ends (see scheme in Scheme 1).[9]

Polymer micelles rank highly among prospective anticancer drug and radionuclide delivery systems, enabling efficient tumor targeting due to their high apparent molecular weight (EPR effect).[10,11] Unimers may be eliminated rapidly from organisms by glomerular filtration after disassembly of the micellar system that fulfilled its task as a drug carrier.[10,11] Many micelles with a thermoresponsive core (made of a polymer block with a lower critical solubility temperature (LCST)) and a hydrophilic corona have the additional advantage of simple preparation by heating of an aqueous solution of the thermoresponsive

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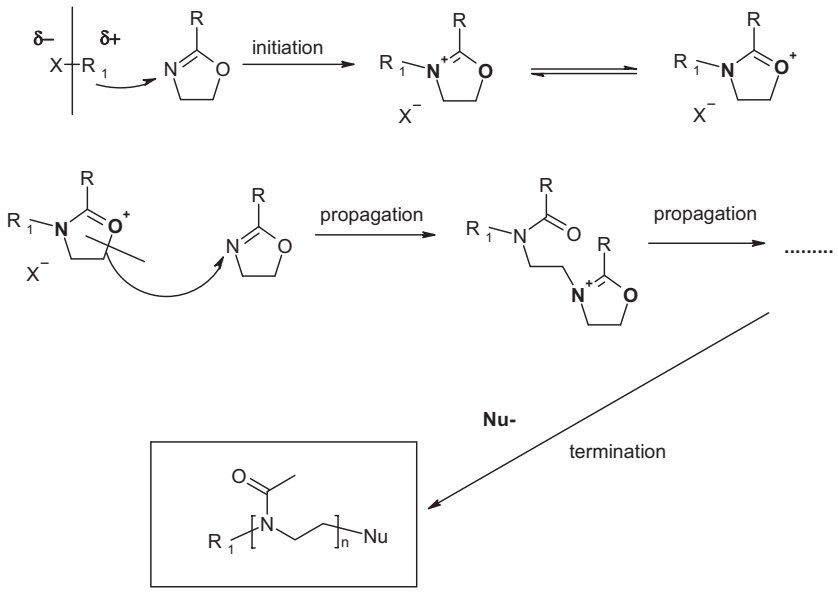



*Scheme 1.* Mechanism of living cationic polymerization of 2-alkyl-2-oxazolines.

polymer.[12] Since there are thermoresponsive poly(2-alkyl-2-oxazolines) with suitable properties described in the literature,[13–17] we decided to use this class of polymers for the construction of our thermoresponsive micellar radionuclide delivery systems.

In this paper we describe the synthesis and study of the properties of ABA triblock copolymer poly[2-methyl-2-oxazoline-*block*-(2-isopropyl-2-oxazoline-*co*-2-butyl-2-oxazoline)-*block*-2-methyl-2-oxazoline] with two hydrophilic A blocks (poly(2-methyl-2-oxazoline)) and one central thermoresponsive B block copolymer (poly(2-isopropyl-2-oxazoline-*co*-2-butyl-2-oxazoline)) with different monomer unit ratios. These polymers are molecularly soluble in aqueous milieu below the cloud point temperature (CPT) of the thermoresponsive block and self-assemble into micelles at higher temperature. Micelles are formed within a narrow temperature range. The CPT of the thermoresponsive block may be adjusted with the 2-butyl-2-oxazoline (hydrophobic monomer lowering the CPT) to 2-isopropyl-2-oxazoline (main monomer giving thermoresponsive properties to its copolymers) ratio and the size of the micelles can be controlled by the A to B block weight ratio. A phenolic moiety was introduced into the above stated polymer to allow radionuclide labeling with iodine radioisotopes for both diagnostics ($^{123}$I, $^{124}$I) and therapy ($^{131}$I) of solid tumors. The polymers were isotopically labeled and the in vitro stability of the radiolabel was checked.

## Experimental Part

### Low Molecular Weight Precursors

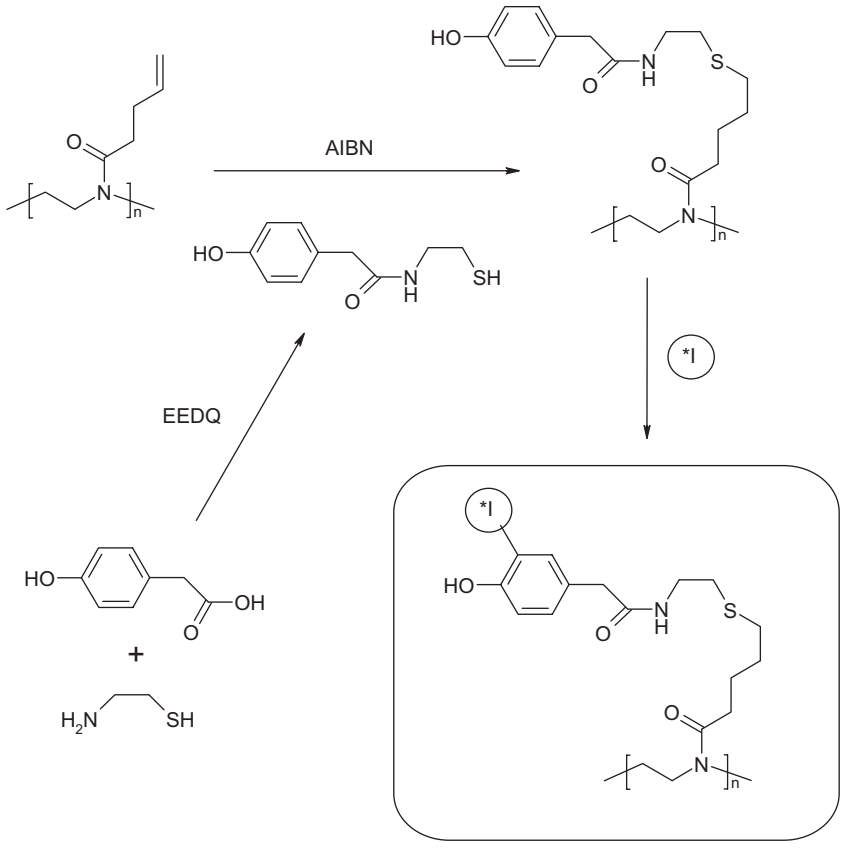
2-Isopropyl-2-oxazoline (IprOX), 2-butyl-2-oxazoline (BuOX) and 2-butenyl-2-oxazoline (EnOX) were synthesized according to ref.[5,18] by condensation of ethanolamine with the particular carboxylic acid (isobutyric or valeric for IprOX and BuOX, respectively), or by a multistep method according to ref. [18] (EnOX). *N*-(2-sulfanylethyl)-2-(4-hydroxyphenyl) acetamide was synthesized by condensation of 4-hydroxyphenylacetic acid and cystamine with (EEDQ) as a condensation reagent according to the following procedure (see Scheme 2). Cysteamine (2.00 g, 25.9 mmol) was dissolved by gentle heating in tetrahydrofuran (THF, 200 mL), 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (6.41 g, 25.9 mmol) was dissolved in THF (50 mL) and 4-hydroxyphenylacetic acid (3.44 g, 22.6 mmol) was also dissolved in THF (20 mL). The solution of EEDQ was mixed with the solution of 4-hydroxyphenylacetic acid and then the solution of cysteamine was added. The mixture was stirred overnight at ambient temperature, heated at 60 °C for 1 h and then evaporated in vacuo. The solid residue was vigorously shaken with water and diethyl ether (aa 100 mL). The suspension was filtered and the collected precipitated product was recrystallized from methanol. The yield was 3.34 g (70%).

$^1$H NMR ($CD_3OD$): $\delta = 2.75$ (t, $-CH_2-S-$), 3.33 (s, Aryl$-CH_2-CO-$), 3.43 (t, $N-CH_2-$), 6.72 (d, Aryl$-$H on positions 2 and 6), 7.08 (d, Aryl$-$H on positions 3 and 5). $^{13}$C NMR ($CD_3OD$): $\delta = 38.4$ ($-CH_2-S-$), 39.7 ($N-CH_2-$), 43.1 (Aryl$-CH_2-$), 116.4 (aromatic carbons in positions 3 and 5), 127.5 (aromatic carbon in position 1), 131.2 (aromatic carbons in positions 2 and 6), 157.5 (aromatic carbon in position 4), 175.0 ($-CO-$). $C_{10}H_{13}NO_2S$ (211.3): Calcd. C 56.85, H 6.20, N 6.63, S 15.18; Found C 56.90, H 6.03, N 6.50, S 15.10.

All other chemicals were purchased from Sigma-Aldrich Ltd. (Prague, Czech Republic). 2-Methyl-2-oxazoline (MeOX) was distilled with calcium hydride before use, and all other chemicals were used as obtained.

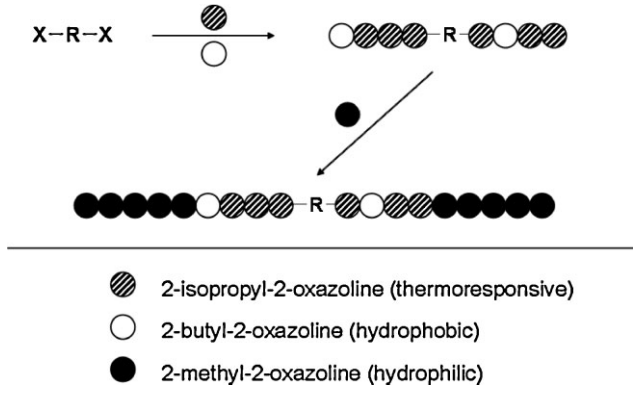
### Synthesis of Polymers

Living cationic ring opening polymerization was performed in acetonitril using tosylates as initiators. Thermoresponsive polymers poly(IprOX-*co*-BuOX) as models of the thermoresponsive central block were synthesized as follows. The mixture of monomers (IprOX with 0, 10 and 20 mol-% BuOX, respectively, total weight 1.00 g in all cases) was polymerized in anhydrous acetonitrile (1.00 mL) using methyl *p*-toluenesulfonate (37 mg, 0.2 mmol) as an initiator. The polymerization was carried out at 42 °C for 8 d in a 15 mL Ace pressure tube (Sigma-Aldrich Ltd., Prague, Czech Republic) under a dry nitrogen atmosphere. The polymerization mixture was mixed with ethanol (30 mL), left overnight at room temperature and evaporated in vacuo. The raw polymer was purified by gel permeation chromatography on Sephadex LH-20 using methanol as the eluent and evaporation of the polymer-containing fractions in vacuo. The polymer was redissolved in ethanol and evaporated in vacuo to obtain solid foam, which was easier to manipulate. A typical yield was ca. 0.90 g (90%).




*Scheme 2.* Scheme of synthesis of sulfanylethyl-2-(4-hydroxyphenyl)acetamide and introduction of isotopically labelable phenolic moiety into the block copolymers.

The triblock ABA copolymers poly[MeOX-*b*-(IprOX-*co*-BuOX)-*b*-2-MeOX] were synthesized as follows in nine alternatives with three different central block compositions (10, 15 and 20 mol-% BuOX, respectively) and thermoresponsive to hydrophilic block ratios (1:2, 1:1 and 2:1 w/w, respectively) in all possible combinations (see Scheme 3). The mixture of 2-isopropyl-2-oxazoline with 10, 15 and 20 mol-% BuOX, respectively, and total weights 0.66, 1.00 and 1.33 g, respectively, was polymerized using diethylene glycol di(*p*-toluenesulfonate) (83 mg, 0.20) as the initiator in anhydrous acetonitrile (1.00 mL). The polymerization was carried out at 42 °C for 7 d in a 15 mL Ace pressure tube (Sigma-Aldrich Ltd., Prague, Czech republic) under a dry nitrogen atmosphere. After this, 2-methyl-2-oxazoline (to the total weight of the sum of oxazoline monomers 2.00 g, i.e., 1.33, 1.00 and 0.66 g, respectively) and anhydrous acetonitrile (1.00 mL) were added and the polymerization continued for 7 d. The polymerization mixture was mixed with ethanol (30 mL) and the mixture was worked up as described above for the polymerization of model poly(IprOX-*co*-BuOX) copolymers. A typical yield was ca. 1.85 g (93%).




*Scheme 3.* Schematic imaging of formation of triblock copolymers.

In the case of the polymers to be isotopically labeled, EnOX (25 mg, 0.20 mmol) was used instead of the equimolar part of BuOX thus forming poly[MeOX-*b*-(IprOX-*co*-BuOX-*co*-EnOX)-*b*-MeOX].

Molecular weights of the polymers were determined by size exclusion chromatography (SEC) in a mixture of acetate buffer (pH 6.5; 0.3 mol · $L^{-1}$) and methanol (20:80 v/v) as a mobile phase on a TSK 3000 column (Polymer Laboratories Ltd., UK) using HPLC System ÄKTA Explorer (Amersham Biosciences; Sweden) equipped with RI, UV and multi-angle light-scattering DAWN DSP-F (Wyatt, USA) detectors. The refractive index increments of polyMeOX ($dn/dc = 0.181 \pm 0.001$ mL · $g^{-1}$) and polyIprOX ($dn/dc = 0.177 \pm 0.002$ mL · $g^{-1}$) in the mobile phase used for molecular weight determination were measured on a Brice-Phoenix visual laboratory type differential refractometer BP-2000-V (Phoenix Precision Instrument Co., USA), and the $dn/dc$ of the copolymers was calculated as a weighted average reflecting monomeric composition.

The content of BuOX monomeric moiety in the thermoresponsive block poly[IprOX-*co*-BuOX] was calculated according to Equation (1) from the $^1$H NMR spectra measured in $CDCl_3$.

$$w_1 = S_{0.91}/(S_{0.91} + (S_{1.09}/2)) \quad (1)$$

where $S_{0.91}$ is the integral signal of the terminal $-CH_3$ hydrogen nuclei in the BuOX monomeric unit at $\delta = 0.91$, $S_{1.09}$ is the integral signal of the $-CH_3$ hydrogen nuclei in the IprOX monomeric units at $\delta = 1.09$ ppm and $w_1$ is the mole fraction of the BuOX monomeric unit in the thermoresponsive block.

The content of the MeOX monomeric moiety in the copolymer was calculated according to Equation (2) from the $^1$H NMR spectra measured in $CDCl_3$.

$$w_2 = (S_{2.09}/3)/((S_{3.46}/4) - (S_{1.09}/6)) \quad (2)$$

where $S_{2.09}$ is the integral signal of the $-CO-CH_3$ hydrogen nuclei in the MeOX monomeric unit at $\delta = 2.09$, $S_{3.46}$ is the integral signal of the oxazoline backbone $N-CH_2-CH_2-N$ hydrogen nuclei at



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$\delta = 3.46$ ppm, $S_{1.09}$ is the integral signal of the $-CH_3$ hydrogen nuclei in the IprOX monomeric units at $\delta = 1.09$ ppm and $w_2$ is the mole fraction of the MeOX monomeric unit in the copolymer.

### Addition of *N*-(2-Sulfanylethyl)-2-(4-hydroxyphenyl)acetamide to Double Bonds of Poly[MeOX-*b*-(IprOX-*co*-BuOX-*co*-EnOX)-*b*-MeOX]

Poly[MeOX-*b*-(IprOX-*co*-BuOX-*co*-EnOX)-*b*-MeOX] (100 mg) and *N*-(2-sulfanylethyl)-2-(4-hydroxyphenyl)acetamide (100 mg) were dissolved in *N*,*N*-dimethylacetamide (DMAc, 1.2 mL). Azobis(isobutyronitril) (AIBN, 50 mg) was dissolved in DMAc (800 μL). The solution of polymer and thiol was heated at 100 °C for 24 h while the solution of AIBN was added in four 200 μL aliquots every 2 h (i.e., at $t = 0$, 2, 4 and 6 h). The solution was cooled to ambient temperature, methanol (3.0 mL) was added and the polymer was isolated by gel permeation chromatography on Sephadex LH-20 column using methanol as the eluent and evaporation of the polymer containing fraction. The yield was 82 mg (82%).

The content of the phenolic moiety was determined by $^1$H NMR in $CDCl_3$ according to Equation (3):

$$c_{\text{phenol}} = 2 \times 10^6 \times S_{7.12}/(M \times S_{3.46}) \quad (3)$$

where $S_{7.12}$ is the integral signal of the Aryl-H hydrogen nuclei in the *ortho*- position relative to the hydroxy group on the aromatic ring at $\delta = 7.12$ ppm, $S_{3.46}$ is the integral signal of the oxazoline backbone $N-CH_2-CH_2-N$ hydrogen nuclei at $\delta = 3.46$ ppm, $M$ is the average molecular weight of monomeric units weighted in respect to their content in the copolymer and $c_{\text{phenol}}$ is the phenolic unit content in micromol per gram of polymer.

### Cloud Point Temperature Determination

Cloud point temperatures (CPT) of the polymers were determined in aqueous solutions of the particular polymer (25 mg · mL$^{-1}$) at a heating rate of 1 °C · min$^{-1}$ with laser scattering detection.

### Static (SLS) and Dynamic (DLS) Light Scattering

The temperature-induced micelle formation in aqueous solutions, temperature dependences of the apparent hydrodynamic radius of the particle, $R_h$, and scattering intensity, $I_s$, were automatically measured at a scattering angle $\theta = 173°$ on a Zetasizer Nano-ZS, Model ZEN3600 (Malvern Instruments, UK). For evaluation of data, the DTS (Nano) program was used. It provides a $R_h$ intensity, volume and number-weighted distribution function $G(R_h)$. A volume-weighted value of apparent $R_h$ was chosen for the monitoring of temperature changes in the system because it gives a more realistic view of the characteristic particle size in a solution. Such information is more valuable for assessing possible biomedical impact. The temperature dependences of micelle formation and disintegration (a hysteresis measurement) were measured by heating from 10 to 70 °C and back in variable steps: 0.5 or 1.2 °C (slow temperature variations). After every temperature change, heating or cooling, five measurements were performed after reaching steady state conditions.

The accurate determination of the shape factor of the particle $R_g/R_h$ was carried out by static and dynamic light scattering measurements at a temperature just above a threshold, to avoid multiple scattering influence, in the angular range 30–150° using an ALV instrument equipped with a 30 mW He-Ne laser (vertically polarized light at $\lambda = 632.8$ nm). The light scattering data were taken after a fixed waiting time of 20 min to achieve equilibration of the sample. The Zimm plot procedure was used for the $R_g$ evaluation. Dynamic light scattering measurements were carried out multiple times at a 90° angle. The obtained correlation functions were analyzed by REPES analytical software providing a hydrodynamic radius distribution function, $G(R_h)$. To account for the logarithmic scale on the $R_h$ axis, all DLS distribution diagrams are shown in the equal area representation, $R_hG(R_h)$. The values of $R_h$ for each system were averaged over three runs.

If not otherwise stated, all measurements were done with a polymer concentration of 0.5 g · L$^{-1}$ in water and all solutions were filtered through a 0.22 μm PVDF syringe filter before a measurement.

### Determination of Critical Micelle Concentration (CMC) by Static Light Scattering Measurements

For polymers that have a cloud point temperature below body temperature, CMC values were determined by static light scattering experiments as the intersection points of straight lines drawn through the data at small and large concentrations (see Supporting Information for a typical example). Static light scattering measurements were carried out multiple times at a 90° angle using an ALV instrument equipped with a 30 mW He-Ne laser (vertically polarized light at $\lambda = 632.8$ nm). Heating was achieved by immersion of the polymer solution (ca 1 mL) in a measuring cell placed into a pre-heated thermostated bath (37 °C).

### Radioisotope Labeling Studies

Radioisotope labeling with $^{125}$I and stability studies were performed as described in the literature.[19]

### Haemolytic Assay

Human blood from a healthy volunteer was collected in 5 mL Heparin coated vacutainers (Greiner Bio-One, Austria) and centrifuged for 5 min at 3 000 rpm. The blood sediment was twice washed in fresh PBS (pH 7.4) and 4% human red blood cell (RBC) suspension was prepared. The micellar delivery system was added to RBC suspensions to achieve final concentrations in the range 1–1 000 μg · mL$^{-1}$. All samples were incubated at 37 °C in a humidified 5% $CO_2$ – 95% air atmosphere for either 30 min or 24 h and then centrifuged for 5 min at 3 000 rpm. The supernatants were spectrophotometricaly analyzed at 550 nm (Spectra Rainbow, Tecan, Austria). Distilled water was used as a positive control of haemolysis and PBS as a negative control. The value of haemolytic activity was calculated by considering absorbance of positive

control as 100% haemolysis. The results are the means of two independent experiments, and the standard deviation was lower than 5%.

## Results and Discussion

### Polymer Synthesis

The thermoresponsive polymeric micellar drug delivery system was made of a ABA triblock copolymers poly[MeOX-*b*-(IprOX-*co*-BuOX)-*b*-MeOX] with two hydrophilic A blocks (polyMeOX) and one central thermoresponsive B block [poly(IprOX-*co*-BuOX)] with different monomer unit ratios. The triblock copolymers were synthesized by living cationic ring-opening polymerization using diethylene glycol ditosylate as the initiator. This highly reactive commercially available bifunctional initiator allows synthesis of triblock copolymer by a one pot method in two steps starting with the middle copolymer block. In the first step, the mixture of monomers forming the thermoresponsive block (IprOX + BuOX) is polymerized with diethylene glycol ditosylate forming a thermoresponsive polymer block with two living cationic ends. In the second step, the monomer forming the hydrophilic terminal blocks (MeOX) was added and the polymerization continued on both chain ends forming two terminal hydrophilic polymer chains. The polymerization was done under conditions (anhydrous acetonitrile, 42 °C) described in the literature[2] to keep the polymerization living without significant chain transfer or termination. Copolymerization was set by the initiator-monomer ratio to synthesize copolymers of a theoretical molar weight of 10 kDa. This molar weight is sufficiently low to allow elimination of unimers by kidneys (the renal threshold is ca. 45 kDa for hydrophilic polymers) after disassembly of the system, but still sufficiently high to suppress intermolecular heterogeneity (given by the statistical nature of the IprOX-BuOX copolymerization) among the polymer chains.

Before using central thermoresponsive IprOX-BuOX copolymer for the synthesis of the triblock copolymers, we optimized its composition with respect to the cloud point temperature. IprOX homopolymer itself has relatively high cloud point temperature only slightly below body temperature (33 °C at $c_{polymer} = 25\ mg \cdot mL^{-1}$). This is why it should be decreased to assure formation of micelles at body temperature if also more hydrophilic corona-forming blocks are present in the macromolecule. The hydrophilic blocks significantly increase the micelle-forming temperature compared to the cloud point temperature of the thermoresponsive block alone (see below). The presence of hydrophobic groups (monomer units) in the polymer makes solvation interactions weaker, which results in a decrease in CPT of such copolymers (LCST is an entropy-driven transition, i.e., the release of water in the solvation shell into the bulk water resulting in an entropy gain is the driving force).

We have chosen BuOX as a more hydrophobic monomer for this study. Synthesis of the model thermoresponsive copolymers was carried out under the same conditions as synthesis of the block copolymers (anhydrous acetonitrile, 42 °C). The molar weight was set to be the average value as in the triblock copolymers (5.0 kDa). Methyl tosylate was used as an initiator in this case. The copolymers were obtained in high yields (> 90% after purification), had molar weights in good correspondence with theory (weight-average molecular weights $\overline{M}_w = 4.72$ kDa in the range $\pm 0.19$ kDa) and narrow molecular weight distributions (polydispersities $I = \overline{M}_w/\overline{M}_n = 1.15$ in the range $\pm 0.02$, where $\overline{M}_n$ is the number-average molecular weight); see Supporting Information for a typical size-exclusion chromatogram. The content of the BuOX monomeric unit in the copolymers is within the experimental error identical (correlation coefficient 0.997, i.e., within 15 relative % of the theoretical value in all cases) with the composition of the polymerization mixture, consistent with the nearly quantitative polymer yield and thus nearly complete monomer consumption. The monomer unit composition in the copolymer was followed by $^1$H NMR (see Experimental Part for details and Supporting Information for typical NMR). An increase in the content of BuOX in the copolymers within a range 0–20 mol-% of BuOX causes a nearly linear ($R^2 = 0.996$) decrease in their CPT (0.81 °C per mol-% BuOX). The cloud point temperature was slightly higher at lower copolymer concentrations in a solution and this concentration dependence was somehow more pronounced in statistic IprOX-*co*-BuOX copolymers compared to pure IprOX homopolymer (see Supporting Information), probably due to statistical intermolecular heterogeneity (similar to that reported for other polyoxazoline copolymers).[20,21]

The triblock copolymers poly[MeOX-*b*-(IprOX-*co*-BuOX)-*b*-MeOX] were synthesized in high yields (> 90% after purification). Their molar weights were only slightly lower than theoretically expected (found $\overline{M}_w = 8.58$ kDa in the range $\pm 0.34$ kDa) with slightly higher polydispersities ($I = 1.39$ in the range $\pm 0.04$) compared to the model copolymers poly(IprOX-*co*-BuOX); see Supporting Information for a typical size-exclusion chromatogram. The slightly higher polydispersities may be due to the use of ethylene glycol ditosylate as an initiator, which results in slower initiation compared to methyl tosylate which yields broader molar mass distributions. This is comparable to the recently reported difference using butyne tosylate and propargyl tosylate initiators.[22] Monomeric unit composition, as followed by $^1$H NMR (see Experimental Part for details and Supporting Information for typical NMR) was identical within the experimental error with the monomer feed (correlation coefficient 0.996 within 15 relative-% of the theoretical value in all cases), consistent with the near



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quantitative conversion, as was also the case for the model copolymers.

### Temperature-responsive Aggregation Behavior

Static and dynamic light scattering methods are common tools to characterize temperature-dependent structural changes of thermoresponsive polymers with LCST. The intensity of the scattered light is sensitive to the molar mass and the size of the scatters and thus can be used to follow the phase separation. A detailed review of the properties of temperature-sensitive polymers, including light scattering studies, has been published.[23] Recently, light and neutron scattering methods were exploited extensively for the investigation of these polymers, e.g., of poly(*N*-isopropylacrylamide)[24,25] (PNIPAM), poly(*N*-vinyl caprolactam)[24,26] (PVCL), poly(methyl vinyl ether)[24] (PVME), hydrophobically modified poly(*N*-isopropylacrylamide) (HM-PNIPAM)[27–31] or of hydrophobically modified polyoxazolines.[32–34]

Several scenarios for the structural transition in a solution during phase separation could come to life, depending on the chemical composition and concentration of the thermoresponsive polymer. It was proven that PNIPAM with high molecular weight at low concentrations exists in a coil conformation below the LCST. On approaching the LCST, a homopolymer undergoes a coil-globule transition, wherein the loose macromolecules at first collapse into a compact globule with further aggregation of globules into a so-called mesoglobule. The PNIPAM mesoglobules are uniform and colloidally stable particles with some amount of water. Once created, they have a tendency to shrink with increasing temperature. At high concentrations, PNIPAM macromolecules form intermolecular aggregates below the CPT. The propensity to aggregate in cold water below the CPT was found for other thermosensitive homopolymers - PVCL and PVME, disregarding their molecular weight and concentration. Loose and polydisperse aggregates transform above the CPT into nearly monodisperse compact (although non-uniform) mesoglobules with a sponge-like structure. The density of such mesoglobules declines from the center to the periphery. The third scenario is realized when a macromolecule contains chemically different moieties: thermoresponsive/hydrophobic or thermoresponsive/hydrophilic. Inclusion of hydrophilic groups in a macromolecule shifts CPT to higher values; hydrophobic moieties have an opposite effect. Below CPT at low concentrations, hydrophobically modified macromolecules form multi-chain assemblies, presumably flower-like micelles, with low aggregation numbers.[25,27,30–33] At higher concentrations, flower-like micelles form inter-micelle aggregates with lower density. When approaching the CPT, flower-like micelles undergo a coil-to-globule transition and monodisperse aggregates or mesoglobules are formed.[23] It was noticed that the mesoglobules occurring above the CPT adopt a core-shell structure resembling a micelle.

In the majority of cases, the hysteresis in the temperature dependence of apparent $R_h$ is manifested. One can see that the mesoglobules disintegrate at a lower temperature in comparison with the one of creation. The $R_h$ value of the mesoglobules also shows non-monotonous behavior near the CPT. Occurrence of hysteresis was explained by the presence of intra- or interchain hydrogen bonds that are broken during cooling.[23,25]

Our light scattering experiments prove that the synthesized thermosensitive polyoxazolines have similar features to the thermosensitive polymers described above. We have completed a basic characterization in water to make the system as principally transparent as possible. In physiological solution (0.9 wt-% NaCl), the temperature-dependent behavior is exactly the same in shape, but everything is shifted 3 °C to lower temperatures. All samples visually become turbid when heated above CPT where dynamic light scattering reveals the formation of nanosize objects (see Supporting Information for chart). On cooling down the samples to below CPT, turbidity disappears, so a transparent solution is formed. Formation of nanoparticles is completely reversible in all cases. Because the perspective structure of nanoparticles built from the studied polymers will be a core-shell one, we will exploit hereafter the term micelle rather than mesoglobule.

The CPT value is a function of the polymer composition (see Supporting Information for chart and Table 1). One can see that through the whole polymer series the CPT increases as the content of hydrophilic MeOX groups increases. Conversely, increasing the content of hydrophobic BuOX moieties results in CPT reduction.

In all cases, the micelle-formation temperatures of the triblock copolymers are significantly higher than CPTs of the thermoresponsive copolymers of the same composition as the thermoresponsive block of the triblock copolymer. If we also compare triblock copolymers with different thermoresponsiveness to hydrophilic block ratios (but the same ratio of monomeric units in the thermoresponsive block), the increase in the hydrophilic block content considerably increases CPT. One can thus conclude that the overall hydrophilicity/hydrophobicity of the whole triblock copolymer is at least of the same importance as the ratio of monomeric units and subsequently the CPT of the thermoresponsive block itself. These triblock copolymers thus behave more like Pluronics [poly(ethylene oxide-*block*-propylene oxide-*block*-ethylene oxide) block copolymers] or poly(ethylene oxide-*block*-lactide) than like, for example, *N*-isopropyl acrylamide copolymers, where the CPT of the thermoresponsive block itself is dominant in determination of the thermal behavior of the block and graft copolymers. The effect also cannot be attributed to the

*Table 1.* Chemical composition and properties of ABA triblock copolymers poly[2-methyl-2-oxazoline-*block*-(2-isopropyl-2-oxazoline-*co*-2-butyl-2-oxazoline)-*block*-2-methyl-2-oxazoline]; $n_{MeOX}$ - average number of 2-methyl-2-oxazoline monomeric units per polymer chain, $n_{IprOX}$ - average number of 2-isopropyl-2-oxazoline monomeric units per polymer chain, $n_{BuOX}$ - average number of 2-butyl-2-oxazoline monomeric units per polymer chain.

| Triblock colymer | Thermoresponsive to hydrophilic block | BuOX in thermoresponsive block | $T_{dem}$ | CMC at 37 °C | $n_{MeOX}$ | $n_{IprOX}$ | $n_{BuOX}$ |
|---|---|---|---|---|---|---|---|
| | w/w | mol-% | °C | $g \cdot mL^{-1}$ | | | |
| P2574-1:2 | 1:2 | 10 | 62 | – | 78 | 27 | 2.6 |
| P2574-1:1 | 1:1 | 10 | 63 | – | 59 | 40 | 3.9 |
| P2574-2:1 | 2:1 | 10 | 64 | – | 39 | 53 | 5.2 |
| P2639-1:2 | 1:2 | 15 | 44 | – | 78 | 25 | 3.9 |
| P2639-1:1 | 1:1 | 15 | 28 | $1.0 \times 10^{-4}$ | 59 | 38 | 5.9 |
| P2639-2:1 | 2:1 | 15 | 27 | $2.5 \times 10^{-5}$ | 39 | 50 | 7.9 |
| P2620-1:2 | 1:2 | 20 | 42 | – | 78 | 24 | 5.2 |
| P2620-1:1 | 1:1 | 20 | 38 | – | 59 | 35 | 7.9 |
| P2620-2:1 | 2:1 | 20 | 28 | $5 \times 10^{-5}$ | 39 | 47 | 10.5 |

effect of the molecular weight of the thermoresponsive blocks, because the molecular weights of the model thermoresponsive copolymers and of the thermoresponsive block in the triblock copolymers are comparable.

Below the CPT, the values of the hydrodynamic radius of the P2639-2:1 polymer correspond to the size of single macromolecules (1–2 nm average), implying that that they are fully dissolved (Figure 1(a)). The scattered light intensity increases approaching the CPT (Figure 1(b)). At the CPT it sharply increases and a simultaneously increasing apparent $R_h$ indicates the formation of micelles (Figure 1).

Although all the triblock copolymers form micelles at certain temperatures, their properties above the CPT differ, depending on the ratio between hydrophobic, hydrophilic and thermosensitive moieties. Thus for P2620-1:1 polymer with the highest content of hydrophobic BuOX groups (20 mol-% in the thermosensitive block) and medium hydrophilicity, it was observed that the nanoparticle size continuously decreases with increasing temperature (Figure 2(a)). Meanwhile, the intensity of the scattered light grows (Figure 2(b)). Heating and cooling measurements shows minor hysteresis of about 2 °C (see Figure 2(a)). In contrast to that, for solutions of P2639-1:2, the intensity and size of the micelles above the CPT permanently grows (Figure 3). Heating and cooling measurements show almost complete absence of hysteresis. Hydrophobic/hydrophilic interactions among whole macromolecules (as a result of the overall hydrophilicity/hydrophobicity of the polymer chains) thus become dominant above CPT for the P2639-1:2 polymer compared to polymer P2620-1:1 due to the shorter length and lower hydrophobicity of the thermoresponsive block (a switch from block polymer-type behavior shown in Figure 2(a) and 2(b)).

The trend of changes during heating and cooling in apparent $R_h$ for P2639-2:1 is quite different to those of P2639-1:2 and P2620-1:1 (see Supporting Information for chart). The apparent $R_h$ dramatically increases above the cloud point and the sharp peak in the $R_h$ vs. temperature dependence reveals the formation of large intermolecular aggregates. At higher temperatures, aggregates gradually shrink and micelles are formed above 40 °C. The shape factor ($R_g/R_h$) values at temperatures 15–20 °C above the CPT are approximately 0.8–1. Minor hysteresis is detectable for the polymer (see Supporting Information for chart). Such behavior is in agreement with previous observations for thermoresponsive polymers reported

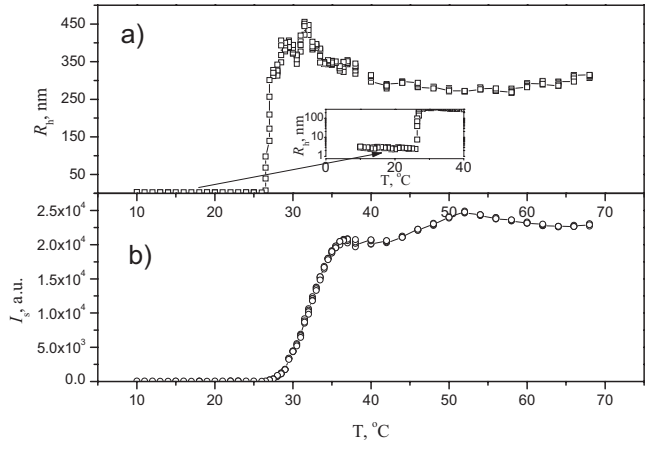


*Figure 1.* Temperature dependence of volume-weighted $R_h$ (a) and the intensity of scattered light $I_s$ (b) of P2639-2:1 polymer at $\theta = 173^{\circ}$, cooling ($c_P = 0.5\ g \cdot L^{-1}$).



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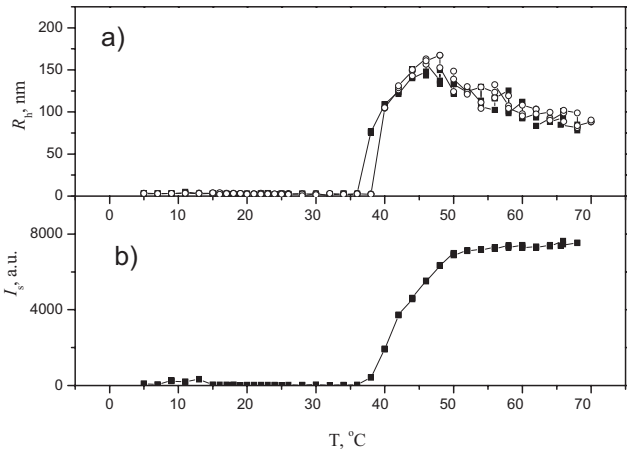


*Figure 2.* Temperature dependence of volume-weighted $R_h$ (a) and the intensity of scattered light $I_s$ (b) of P2620-1:1 polymer at $\theta = 173^\circ$, heating (○) and cooling (■) ($c_P = 0.5\ g \cdot L^{-1}$).

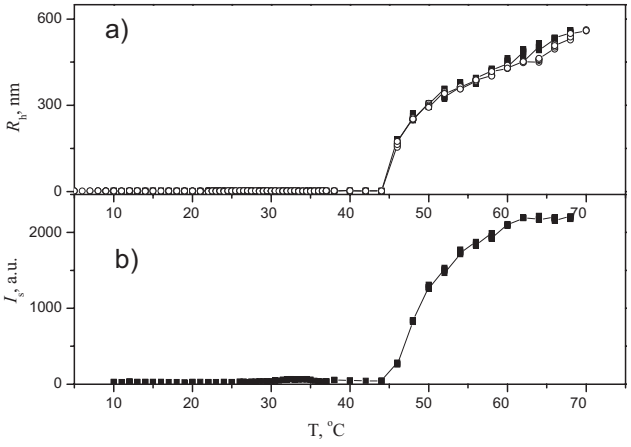


*Figure 3.* Temperature dependence of volume-weighted $R_h$ (a) and the intensity of scattered light $I_s$ (b) of P2639-1:2 polymer at $\theta = 173^\circ$, heating (○) and cooling (■) ($c_P = 0.5\ g \cdot L^{-1}$).

in the literature.[23] It is reasonable to assume that due to the smallest content of hydrophilic MeOX groups in the macromolecular structure, the thermosensitive IprOX block have control over behavior of the aggregates above the CPT.

A very important property of micelles designed for drug delivery purposes is their critical micellar concentration (CMC). The micelles must be sufficiently stable in dilution in the bloodstream (total blood volume in humans is ca. 5 L). They should not contain too much unimer in equilibrium with the micelles, since hydrophobic domains of unimers are not protected from unwanted interactions in the body by the hydrophilic corona and unimers may also be quickly eliminated through the kidneys due to their relatively low molecular weight. On the other hand, too stable micelles may have a problem with a too slow rate of elimination from the organism. The CMC values for the polymers (Table 1, since the measurements were carried out at 37 °C, only polymers forming micelles at this temperature were measured) are somehow higher but comparable with the CMC obtained with purely amphiphilic block and graft copolymers, in accordance with the literature. As mentioned above, this may lead to shorter blood circulation times, but, on the other hand, should facilitate polymer elimination from the organism after the system fulfills its task.

## Radionuclide Labeling

Since copolymer micelles are intended as carriers for radiodiagnostics/radiotherapeutics and polyoxazolines without suitable functional moieties cannot be directly radiolabeled, we introduced radiolabelable phenolic moiety into the copolymers. Phenol is a highly activated aromatic moiety towards electrophilic radioiodination with iodine radioisotopes suitable for radiodiagnostics ($^{123}I$, $^{124}I$) or radiotherapy ($^{131}I$). We have chosen the polymer P2639-1:1 containing 15 mol-% BuOX in the thermoresponsive block and a 1:1 thermoresponsive to hydrophilic block ratio as a starting copolymer for further modification due to its suitable properties (micelle-forming temperature and $R_H$, see Table 1 and Supporting Information). The radiolabelable moiety was introduced into the triblock copolymer by the introduction of pendant double bonds into the copolymer (part of BuOX in the polymerization mixture was substituted with EnOX to achieve on average one double bond per polymer molecule) and thiol-clock addition in analogy to ref.[18] of sulfanylethyl)-2-(4-hydroxyphenyl)acetamide onto the double bond (see Scheme 2). In analogy to ref.[35], AIBN was used as an initiator instead of UV-light. UV-light photocatalysis was originally described for another thiol-ene click reaction to polyoxazolines,[36] but the photoinitiated reaction offered unsatisfactory conversions only in our case. No polymer-bound initiator fragments can be detected by NMR since there is an excess of the thiol in the reaction mixture. The achieved phenolic moiety content ($36.2\ \mu mol \cdot g^{-1}$ polymer) and thus the theoretical labeling capacity (61 GBq $^{125}I$/mg polymer) calculated from the phenol content assayed by $^1H$ NMR was more than sufficient for the possible application (the typical dose per human patient is 0.02–3 GBq depending on the particular radionuclide and application).

Radionuclide labeling was carried out using the chloramine method in good yield (66%). Most of the radioiodine was bound to polymer by a stable bond (see Supporting Information for a chart) and the remaining part was gradually released into low molecular weight fraction during incubation in PBS buffer at 37 °C. This means that part of the iodine is bound by a metastable bond; however, if GPC separation on a PD-10 desalting column is repeated after 2.5 h, a more stable product is obtained and should be sufficient for the intended radiodiagnostic purposes.

Haemolytic activity is a straightforward measure of eventual membrane toxicity due to the amphiphilic character of the copolymers. All the copolymers have shown no toxicity (haemolysis less than 3.5% even at the highest concentration used ($1\,mg \cdot mL^{-1}$) which corresponds to the hypothetical total dose of 5 g per human with a total blood volume of 5 L, (see Supporting Information for a chart).

## Conclusion

We have synthesized ABA triblock copolymers poly[2-methyl-2-oxazoline-*block*-(2-isopropyl-2-oxazoline-*co*-2-butyl-2-oxazoline)-*block*-2-methyl-2-oxazoline] with two hydrophilic A blocks and one central thermoresponsive B block with different monomer unit ratios. These polymers are soluble in aqueous millieu, molecularly dissolved below the cloud point temperature of the thermoresponsive block and form micelles at higher temperature. Micelles are formed within a narrow temperature range. The CPT of the thermoresponsive block was adjusted with 2-butyl-2-oxazoline (hydrophobic monomer lowering the CPT) to 2-isopropyl-2-oxazoline (main monomer giving thermoresponsive properties to its copolymers) ratio and size of the micelles was also influenced by the A to B block weight ratio. A phenolic moiety was introduced into the above stated polymer to allow radionuclide labeling with iodine radioisotopes for both diagnostics and therapy of solid tumors. Such polymer was then radiolabeled with $^{125}I$ in good yield with sufficient in vitro stability under model conditions.

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